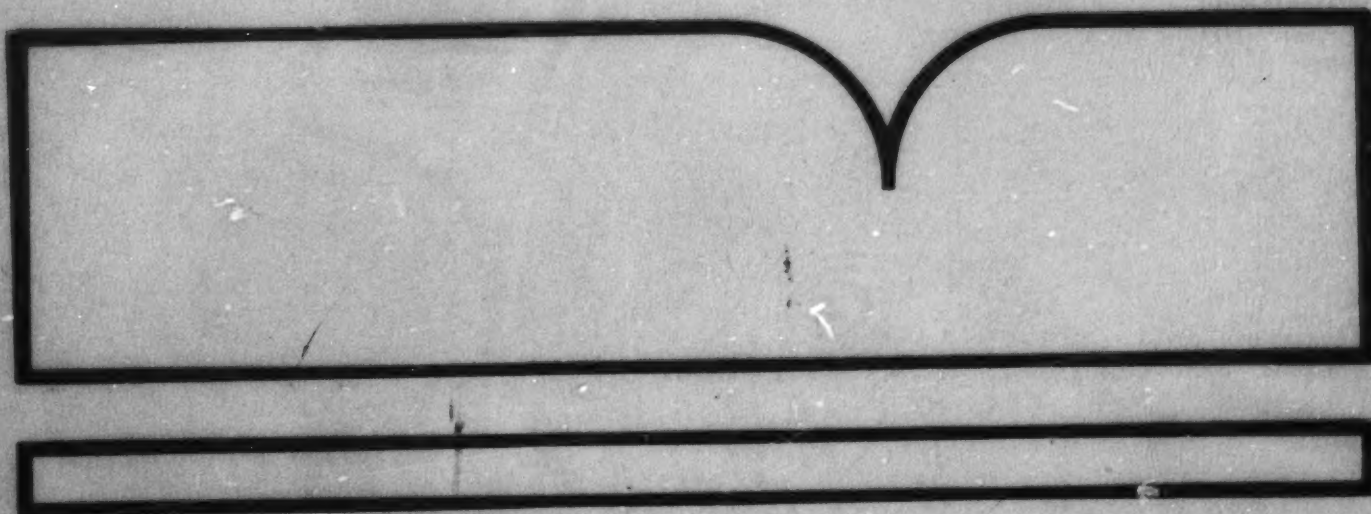


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Early Life-Stage Toxicity Test with Tidewater  
Silversides (*Menidia peninsulae*) and  
Chlorine-Produced Oxidants

(U.S.) Environmental Research Lab.  
Gulf Breeze, FL

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Short Communication

EARLY LIFE-STAGE TOXICITY TEST WITH TIDEWATER  
SILVERSIDES (*MENIDIA PENINSULAE*) AND CHLORINE-  
PRODUCED OXIDANTS

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**Abstract** — Early life-stage toxicity tests (continuous exposure from embryonic stage to 3 weeks or more into the exogenous feeding stage) with North American marine fishes have been conducted almost exclusively with cyprinodontids. In this report, we present methods for testing a representative of another family, Atherinidae. Embryos of the tidewater silverside *Menidia peninsulae* (Goode and Bean) were obtained by a laboratory spawning procedure that required lighting and tidal (current) stimuli. A 28-d toxicity test with chlorine-produced oxidants (CPO) began with stage 21 and 22 embryos (approximately 36 h old). Average measured CPO concentrations in exposure water were nondetectable ( $< 0.01$  mg/L) in the control and in the two lowest exposure concentrations, and 0.01, 0.04 and 0.21 mg/L. Survival of embryos to hatching averaged 99%, with no significant difference among treatments. Although no fish survived exposure to 0.21 mg CPO/L, survival was  $\geq 88\%$  in the control and the four other CPO treatments. Average wet weights of individual fish ranged from 11.7 mg in 0.04 mg/L to 13.2 mg in 0.01 mg/L, with no significant difference among treatments.

**Keywords** — Chlorination    Chronic toxicity    *Menidia peninsulae*    Early life stage

INTRODUCTION

Data from acute, early life-stage and life-cycle toxicity tests with fish are an integral part of the assessment of the hazard of substances to aquatic life. Chronic testing

procedures have been developed for several North American freshwater fishes [1-4] and an extensive toxicity data base has been produced. In contrast, definitive methods for conducting early life-stage and life-cycle toxicity tests are available for only one estuarine fish, the sheepshead minnow (*Cyprinodon variegatus*) [5-7]. Use of this one species to determine the chronic sensitivity of marine fishes to toxicants may pose problems in that (a) geographical range and habitat preference could limit the availability of test fish and applicability of test results

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and (b) data obtained with this species alone might not accurately predict the responses of other fishes with dissimilar sensitivities.

During the last 4 years, we have been developing chronic toxicity test methods for additional species of marine fishes. In this report, we present techniques for laboratory spawning of the tidewater silverside, *Menidia peninsulae* (Goode and Bean), an early life-stage toxicity test method for this fish and test results obtained with chlorine-produced oxidants (CPO). The tidewater silverside is an estuarine species typically found at salinities  $> 20‰$ . Distribution is disjunct, extending from Daytona Beach, FL, to Horn Island, MS and from Galveston Bay, TX, to Tamiahua, northern Veracruz, Mexico [8]. Little definitive information is available on the natural history of *M. peninsulae* because the species has, until recently, been considered the same as *M. beryllina* [8, 9]. Both species form schools and are undoubtedly important forage for larger fishes.

Chlorine is widely used as a biocide for treating drinking water and wastewater, for controlling fouling organisms in power plants, and as a bleaching agent [10]. A large amount of research has been conducted to determine the effects of chlorination on aquatic organisms. Because of the quantity of literature, differences in chlorine measurement and experimental techniques, and chemical and toxicological complexities, the reader is referred to review papers on the subject [e.g., 11–15].

#### MATERIALS AND METHODS

Sexually mature *M. peninsulae* were collected from Santa Rosa Sound, FL, in the vicinity of our laboratory and maintained in two circular fiberglass tanks (diameter 1.3 m, water depth 35 cm) with a recirculating filter-reservoir system. Thirty adult fish were placed in each tank. Water temperature was maintained at  $24 \pm 1^\circ\text{C}$  and salinity at 22 to 27‰. After being held under continuous lighting and at a current velocity of  $8 \text{ cm s}^{-1}$  for 2 weeks, a photoperiod of 13

h light:11 h dark (lights abruptly on and off) was introduced. Interruptions of current velocity from  $8$  to  $0 \text{ cm s}^{-1}$  were introduced twice daily (0600 to 0700 and 1800 to 1900 h) to simulate slack tides. In response to these nocturnal and tidal flux cues, laboratory-held *M. peninsulae* spawned within a few hours after the lights were turned off. Eggs, approximately 0.9 mm in diameter, were deposited on polyester aquarium filter fiber suspended at the surface of each spawning tank. Fertilized eggs were removed each morning. A detailed description of the recirculating holding system and analysis of spawning cues have been reported by Middaugh and Hemmer [16]. Except for the photoperiod (21 h light:3 h dark), which was being manipulated to investigate its effect on spawning, embryos for the chlorine toxicity test were obtained under the conditions recommended above.

Identification of our *M. peninsulae* spawning stocks was based on the horizontal distance between the origin of the spinous dorsal and anal fins, which was one of the diagnostic characters identified by Chernoff et al. [8]. This distance in our fish ranged from 0.064 to 0.108 ( $\bar{x} = 0.087$ ) of the standard length.

Seawater used in the toxicity test was pumped from Santa Rosa Sound, FL, through a sand filter and a  $20\mu\text{m}$  polypropylene filter, diluted with freshwater to a nominal 20‰ salinity (actual range, 15–22‰) and delivered to an aerated fiberglass reservoir (approximately 9.5 kl). The fresh water used to dilute the filtered seawater (salinity range, 17.5–29‰) was obtained from a chlorinated municipal well water supply, filtered through  $20\mu\text{m}$  polypropylene and charcoal filters and delivered to a similarly aerated reservoir prior to usage. Salinity-adjusted water was automatically delivered as needed to a constant-head box, where it was vigorously aerated and cooled to  $25 \pm 2^\circ\text{C}$ . Water flowed to the exposure aquaria with a diluter similar to that described by Schimmel et al. [17]. At each cycle of the diluter (approximately every 15

min), 1 liter of water flowed to the seawater control and to each of the five treatments containing CPO. Laboratory-grade (4–6%) sodium hypochlorite from Fisher Scientific Co. (Pittsburgh, PA) was used as the stock solution. It was infused into the diluter with a Hamilton Precision Liquid Dispenser II (Reno, NV) at a rate that provided nominal concentrations of 0.0 (seawater control), 0.22, 0.45, 0.89, 1.8 and 3.6 mg/L. Water was delivered from the diluter to splitter boxes equipped with two glass capillary tubes having an internal dimension (i.d.) of 2 mm. One glass tube slowly delivered 0.5 liters to each of two exposure aquaria for each treatment. Water depth in each aquarium (i.d., 22 cm long  $\times$  22 cm wide  $\times$  10 cm high) was varied between  $\sim$  4.5 and 7 cm by a self-starting siphon (4 mm, i.d.). Aquaria were partially immersed in a water bath that maintained the test water at  $25 \pm 2^\circ\text{C}$ . The photoperiod during testing was 12 h light:12 h dark.

Tidewater silverside embryos, approximately 36 h old and at stages 21 to 22 in development [18], were gently teased from the filter fiber spawning substrate and their chorionic fibrils removed by clipping with scissors. Embryos were examined microscopically, and five were placed randomly in each of the 24 incubation cups. This process was repeated until each cup contained 15 embryos. Concentration and replicate number were then assigned to the cups using a set of random numbers. Incubation cups were glass Petri dish bottoms (9 cm, i.d.) with 10-cm-high cylinders of 363  $\mu\text{m}$  nylon mesh attached to the inside walls with silicone adhesive. Embryos were not chemically analyzed, but a sample of the brood fish contained no detectable ( $<0.1 \mu\text{g/g}$ ) organochlorine insecticides or polychlorinated biphenyls.

Embryos and hatched fish were observed daily for deaths and other signs of poisoning, such as abnormal behavior, coloration or feeding rates. Incubation cups were removed from the aquaria when making observations, except on days 4 through 9,

when larvae would have easily been injured. To minimize the risk of stranding or injuring fish on the nylon mesh, water in the aquaria was drained to below the mesh with 3-mm i.d. glass siphons before the cups were removed.

Three feeding regimens were used during the test: rotifers (*Brachionus plicatilis*) for small, newly hatched fish, a transition mixture of rotifers and *Artemia*, and then *Artemia* only. On day 5 of exposure, newly hatched larval silversides were fed rotifers in the morning and afternoon. On days 6 through 10, the fish were fed rotifers three times daily. On days 11 through 13, fish were fed both live *Artemia* (24 to 32 h post-hydration of cysts) and rotifers in a total of two or more feedings each day. On days 14 through 27, the fish were fed *Artemia* twice daily. They were not fed on day 28, when the experiment was terminated. Approximately 7,000 to 10,000 rotifers were provided per feeding per cup. The number of *Artemia* per feeding was quantified only on day 13 (2,000 per cup) when rotifers were also being provided. Considerably more *Artemia* were provided per feeding from day 14 through 27. At the termination of the experiment, fish were killed in hot water, towel drained and weighed individually.

CPO (free and combined chlorine, and other chlorine-produced oxidants) concentrations in test water were determined two to three times weekly using a Wallace and Tiernan (Belleville, NJ) amperometric titrator. Chlorine demand-free water was used to prepare the 5% KI solution, which was refrigerated except when in use. Water samples were prepared for titration according to the procedure outlined by Crecelius et al. [19]. Two milliliters of pH 4 buffer was added to the sample vessel and then 2 ml of KI solution. These reagents were swirled briefly, 200 ml of exposure water was added and the sample was titrated with phenylarsine oxide within  $\sim$  1 min. Average measured CPO concentrations are given in Table 1. Nominal chlorine concentrations (as sodium hypochlorite) and average

Table 1. Survival and average weight of tidewater silversides (*Menidia peninsulae*) continuously exposed to CPO for 28 d at 25°C in an early life-stage toxicity test

| Nominal          | Exposure concentration (mg/L) |                    | Survival (%) |                   |                | Average Weight (mg) |
|------------------|-------------------------------|--------------------|--------------|-------------------|----------------|---------------------|
|                  | Average measured              | Standard deviation | Embryos      | Fry               | Combined       |                     |
| Seawater control | ND <sup>a</sup>               | —                  | 100          | 88.3              | 88.3           | 13.0                |
| 0.22             | ND                            | —                  | 100          | 93.3              | 93.3           | 13.0                |
| 0.45             | ND                            | —                  | 100          | 88.3              | 88.3           | 12.9                |
| 0.89             | 0.01 <sup>b</sup>             | —                  | 96.7         | 98.2 <sup>c</sup> | 95.0           | 13.2                |
| 1.8              | 0.04                          | ± 0.04             | 98.3         | 95.0              | 93.3           | 11.7                |
| 3.6              | 0.21                          | ± 0.13             | 98.3         | 0 <sup>c</sup>    | 0 <sup>c</sup> | —                   |

<sup>a</sup>ND, none detected (<0.01 mg CPO/L).

<sup>b</sup>Five of the 11 water samples contained no detectable (<0.01 mg/L) CPO and were not included in the mean.

<sup>c</sup>Significantly different from control response ( $\alpha = 0.05$ ).

measured CPO concentrations differed substantially, probably as a result of the "chlorine demand" of natural saline waters. This demand results from the occurrence of dissolved and suspended organics and from the presence of bromide, which is oxidized to bromine [20–22].

Dissolved oxygen concentrations were determined weekly in each treatment containing live fish, and averages from all treatments ranged from 6.8 to 6.9 mg/L (overall range, 6.5–7.1 mg/L). Test-water pH in each treatment was determined on day 13, and ranged from 7.9 to 8.0. Suspended solids in our dilution water were determined on day 28 and averaged 1.6 mg/L ( $n = 3$ , range, 0.5–3.7 mg/L).

Analysis of variance and Duncan's multiple range test were used to analyze survival and weight data. Percentages were examined with and without arcsine transformation before analyses. The significance level was  $\alpha = 0.05$ .

#### RESULTS

CPO did not affect survival of embryos but did affect survival of hatched *M. peninsulae*. Embryo survival to hatching averaged 98.9% (range by treatment, 96.7–100%), with no significant differences among the treatments (Table 1). Hatching began on day 4 (one fish) and ended by day 7. At an average measured CPO concentration of 0.21 mg/L, 87% of the fry were dead within 4 d after hatching began; none

survived beyond 10 d after hatching. Combined survival of embryos and fry in all other treatment was  $\geq 88\%$ . Although fry survival at an average measured concentration of 0.01 mg CPO/L was significantly greater than the control response, these two treatments did not differ significantly when combined survival of embryos and fry were analyzed.

There were no significant differences in sublethal responses of fish attributable to CPO exposure. Although the average wet weight of fish exposed to 0.04 mg CPO/L (11.7 mg) was less than that of control fish (13.0 mg), the difference was not statistically significant (Table 1). Furthermore, no consistent signs of poisoning were observed at sublethal CPO concentrations ( $\leq 0.04$  mg/L) that would allow separation of control versus exposed animals.

#### DISCUSSION

Our research demonstrates that *M. peninsulae* can be spawned in the laboratory and used to conduct early life-stage toxicity tests. Overall survival in the control and in CPO concentrations at which no significant adverse effects were observed averaged 92% (embryos, 99%; hatched fish, 93%). Two of the most important factors in ensuring adequate survival of this species during testing are proper feeding and minimal handling, especially in the first few days after hatching. Rotifers appear to be a

satisfactory first food. Approximately 5 d after hatching, the silversides were capable of ingesting *Artemia* nauplii (24–32 h post-hydration of cysts). Since young silversides are susceptible to stranding and injury on mesh in the incubation cups, flow rates through the exposure system must be slow. To prevent injury, the fish must never be netted. We have also conducted experiments that demonstrate that this toxicity test method will work equally well with the Atlantic silverside (*Menidia menidia*).

CPO were lethal to all *M. peninsulae* fry exposed to an average measured concentration of 0.21 mg/L, and no significant adverse effects on either survival or growth were observed at the next lower concentration of 0.04 mg/L. The concentration at which no effect was observed was 74% of the 96-h  $LC_{50}$  of 0.054 mg CPO/L (95% confidence interval, 0.044–0.062 mg/L) obtained with juvenile *M. peninsulae* in a flow-through test with measured concentrations and conducted at our laboratory (J. R. Clark, unpublished data).

Although we are aware of no data on the sensitivities of other marine fishes to CPO in early life-stage toxicity tests, several investigators have determined the acute effects of CPO on selected developmental stages. It should be pointed out that the accepted terminology for combined (as chloramines) plus free chlorine in freshwater is total residual chlorine (TRC). The term TRC has been used by many investigators to describe combined plus free chlorine in saline waters. However, since chlorine added to seawater may form chlorinated and brominated compounds, the term chlorine-produced oxidants (CPO) is now used to describe oxidative products measured amperimetrically at pH 4 in saline waters [23]. The two terms may be considered analogous when comparing our results for CPO with those of investigators using the term TRC.

The effects of chlorine exposure on embryonic survival and larval development of estuarine fishes were investigated by

Morgan and Prince [24,25]. Acute (24–80 h)  $LC_{50}$  values for embryos of five species of estuarine fishes (*Morone saxatilis*, *M. americana*, *Alosa aestivalis*, *Menidia menidia* and *M. beryllina*) ranged from 0.20 to 0.38 mg TRC/L [24]. The  $LC_{50}$  values (24 and 48 h) for various embryonic stages of the two *Menidia* species ranged from 0.21 to 0.38 mg/L (from their Table 1). In our experiment, which began with older embryos, survival of *M. peninsulae* embryos was not significantly reduced by 0.21 mg CPO/L. Continuous exposure of *M. saxatilis*, *M. americana* and *A. aestivalis* embryos to low TRC concentrations significantly reduced larval lengths at hatching [25]. Approximate TRC concentrations (from their Fig. 1) that significantly reduced lengths were  $\geq 0.05$  mg/L for *M. saxatilis*,  $\geq 0.1$  mg/L for *M. americana* and  $\geq 0.25$  mg/L for *A. aestivalis*. In our experiment, lengths of larvae at hatching were not determined, but we found no significant differences in average weights of fish exposed to CPO concentrations  $\leq 0.04$  mg/L at the end of the 28 d exposure that began with embryos.

Alderson [26] found that plaice (*Pleuronectes platessa*) embryos were more resistant to chlorine than were newly hatched larvae, and the acute sensitivity of both plaice and dover sole (*Solea solea*) decreased with increasing larval stage up to metamorphosis. Alderson also found that growth of both species was not markedly reduced by chlorine unless the concentration was close to the lethal concentration. Although we tested a different species and our experimental design was quite different, our results tend to agree with those of Alderson. Survival of *M. peninsulae* embryos to hatching was 98% in our highest concentration (0.21 mg CPO/L), but all the resulting fry died within 10 d after hatching. Also, growth of fry in our next lower concentration, 0.04 mg CPO/L, was not significantly different from that of control fish.

In flow-through tests with measured concentrations, Roberts et al. [27] determined the toxicity of chlorine to field-collected

Atlantic silversides (*Menidia menidia*), northern pipefish (*Syngnathus fuscus*) and naked gobies (*Gobiosoma boscii*). Atlantic silversides were the most sensitive fish tested, with a 96-h  $TL_{50}$  of 0.037 mg TRC/L, which is slightly lower than our no-observed-effect concentration of 0.04 mg CPO/L for *M. peninsulae*.

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